Title: **MUTATOX TOXICITY TEST**

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## 1.0 OBJECTIVE

This method measures the toxicity of environmental samples to the dark mutant of luminescent bacteria, *Vibrio fischeri*, strain M169, to detect the presence of genotoxic agents. The Mutatox test strain exhibits increased light production when grown in the presence of sub-lethal concentrations of genotoxic agents.

## 2.0 HEALTH AND SAFETY

Personnel should wear lab coats, lab aprons, safety goggles, and chemical resistant gloves when preparing chemical stocks, and when dosing with test chemicals or effluents.

## 3.0 PERSONNEL/TRAINING/RESPONSIBILITIES

This method should be restricted to use by or under the supervision of professionals experienced in toxicity testing.

## 4.0 REQUIRED AND RECOMMENDED MATERIALS

Microbics Model 500 Analyzer
Water bath
Mutatox Medium (2 vials)
Reagent Vial (2 vials)
Pipet bulbs
15 ml syringe

Mutatox Test Reagent
Autoclaved De-ionized Water
Mutatox S-9 Medium (2 vials)
10 ml volumetric pipets
0.6 ml syringe
Timer

| | |
|---|---|
| Phenol | Benzo(a)pyrene |
| DMSO | Toluene |
| Isopropanol | Lab coat |
| Gloves | Repeat Pipettor |
| Test Cuvettes | Analytical Scale |

## 5.0 PROCEDURE

### 5.1 Equipment Preparation

5.1.1 Analyzer and Incubator Preparation

- Plug in the Microbics M500 Toxicity Analyzer.
- Place a cuvette in the REAGENT well.
- Plug in the incubator (water bath) and set it to 35°C. Use a thermometer to verify the water temperature.

5.1.2 Solvent and Positive Control Stocks

Two positive controls should be tested with each Mutatox assay; one direct acting compound and a chemical that requires metabolic activation (S-9).

5.1.2.1 Making Solvent Stock

- Add 2ml of DMSO, 1ml of Toluene, and 1ml of Isopropanol (DTI) into a precleaned solvent vial.
- Date and label the vial.
- To make the 1:10 dilution of 2:1:1 of DTI in DMSO, add 900µl of DMSO and 100µl of 2:1:1 of DTI to a pre-cleaned solvent vial.

5.1.2.2 Making Phenol Stock (10 mg/ml stock solution)

- Weigh out 0.005g of Phenol (located in Rm. 230) into a solvent vial.
- Add 0.5 ml of 1:10 DTI:DMSO.
- Date and label the vial.

5.1.2.3 Making Benzo(a)pyrene Stock(10mg/ml stock solution)

- Weigh out 0.005g of Benzo(a)pyrene (refrigerator in Rm. 405) into a solvent vial.
- Add 0.5 ml of 1:10 DTI:DMSO.
- Date and label the vial.

### 5.2 Test Preparation for Three samples (3 replicates each and a spike)

5.2.1 S-9 Test Preparation

- Place 95 cuvettes in each of 2 labeled cuvette supports as indicated in Table 1.
- Rinse (three times) a 15-ml syringe for the repeat pipettor with distilled water.
- Fill two buckets with ice from the ice machine.
- Add 2.2ml of cold (5°C ± 1°C) autoclaved water to the REAGENT well cuvette using a 10 ml glass pipette. Store the water in an ice bath during test preparation and wait 5minutes before using the autoclaved water.
- Pipette 250µl of media into cuvettes: A1 through A4, A6 through A9, B1 through B4, B6 through B9, C1 through C4, C6 through C9, D1 through D4, D6 through D9, E1 through E4, E6 through E 9, F1 through F4, F6 through F9, G1 through G4, G6 through G9, H1 through H4, H6 through H9, I1 through I4, I6 through I9, and J1 through J4.
- Add 500µl of media to the cuvettes: A5, A10, B5, B10, C5, C10, D5, D10, E5, E10, F5, F10, G5, G10, H5, H10, I5, I10, and J5.
- Add 10µl of solvent (1:10 of DTI:DMSO) to solvent cuvettes: A6 through A10. This is the solvent used for sample preparation. In this case, the samples were prepared in a 1 to 10 dilution of DMSO/Toluene/Isopropanol in DMSO.
- Add 10µl of Benzo(a)pyrene (positive control) to corresponding cuvette plus the spikes (B1 through B5, D5, F5, H5, and J5).
- Add 10µl of sample to corresponding cuvettes ($5^{th}$ cuvette).
- Make 1:2 serial dilutions of positive control and samples by transferring 150µl from "cuvette to cuvette" in S-9 cuvette supports. Mixing exactly 3 times from highest to lowest concentration, e.g. A5 to A4, A4 to A3, A3 to A2, and A2 to A1. Discard the extra volume into a labeled jar.

5.2.2 S-9 Test Initiation

- Rinse (three times) a 0.6ml syringe for the repeat pipettor with deionized water.
- Remove two (2) S-9 media vials from the freezer and remove the seal. Immediately reconstitute the pellet of S-9 media using the cuvette from the ice bath. To do so, place the lip of the cuvette on top of the reagent vial. Then, as quickly as you can, dump the reconstitution solution into the reagent vial.
- Immediately swirl the media vials to dissolve the media. Pour both vials back into the cuvette. Use S-9 Media immediately after hydration and keep media in an ice bath during pipetting.
- Place reagent cuvette in the REAGENT well.
- Mix reagent using the repeat pipettor with the rinsed 0.6ml syringe.
- Immediately transfer 0.6µl of reagent to each cuvette. Do not touch the cuvette

walls or contents with the pipettor tip.

- Note and record time.
- Mix cuvettes by gently shaking each cuvette support block.
- Cover both support blocks with Parafilm® to minimize evaporation and place the blocks into the water bath (35°C) for 45 minutes.
- Start Direct Mutatox media preparation.

5.2.3 Direct Mutatox Media Test Preparation

- Place 95 cuvettes in each of 2 labeled cuvette supports as indicated in Table 2.
- Rinse (three times) a 15-ml syringe for the repeat pipettor with distilled water.
- Add 2.2ml of cold (5°C $\pm$ 1°C) autoclaved water to the REAGENT well cuvette using a 10 ml glass pipette. Store the water in an ice bath during test preparation and wait 5minutes before using the autoclaved water.
- Pipette 250µl of media into cuvettes: A1 through A4, A6 through A9, B1 through B4, B6 through B9, C1 through C4, C6 through C9, D1 through D4, D6 through D9, E1 through E4, E6 through E 9, F1 through F4, F6 through F9, G1 through G4, G6 through G9, H1 through H4, H6 through H9, I1 through I4, I6 through I9, and J1 through J4.
- Add 500µl of media to the cuvettes: A5, A10, B5, B10, C5, C10, D5, D10, E5, E10, F5, F10, G5, G10, H5, H10, I5, I10, and J5.
- Add 10µl of solvent (1:10 of DTI:DMSO) to solvent cuvettes: A6 through A10. This is the solvent used for sample preparation. In this case, the samples were prepared in a 1 to 10 dilution of DMSO/Toluene/Isopropanol in DMSO.
- Add 10µl of phenol (positive control) to corresponding cuvette plus the spikes (B1 through B5, D5, F5, H5, and J5).
- Add 10µl of sample to corresponding cuvettes ($5^{th}$ cuvette).
- Make 1:2 serial dilutions of positive control and samples by transferring 150µl from "cuvette to cuvette" in S-9 cuvette supports. Mixing exactly 3 times from highest to lowest concentration, e.g. A5 to A4, A4 to A3, A3 to A2, and A2 to A1. Discard the extra volume into a labeled jar.

5.2.4 Direct Mutatox Media Initiation

- Rinse (three times) a 0.6ml syringe for the repeat pipettor with deionized water.
- Remove two (2) Direct Mutatox media vials from the freezer and remove the seal. Immediately reconstitute the pellet of Direct media using the cuvette from the ice bath. To do so, place the lip of the cuvette on top of the reagent vial. Then, as quickly as you can, dump the reconstitution solution into the reagent vial.
- Immediately swirl the media vials to dissolve the media. Pour both vials back into

the cuvette. Use Direct Media immediately after hydration and keep media in an ice bath during pipetting.

- Place reagent cuvette in the REAGENT well.
- Mix reagent using the repeat pipettor with the rinsed 0.6ml syringe.
- Immediately transfer 0.6µl of reagent to each cuvette. Do not touch the cuvette walls or contents with the pipettor tip.
- Mix cuvettes by gently shaking each cuvette support block.
- Cover both support blocks with Parafilm® to minimize evaporation
- After 45 minutes for the S-9 test, change the water temperature to 27°C. Verify the temperature with a thermometer and place the Direct media blocks into the water bath.
- After 14, 16, 20, and 24 hours incubation, call up the Microtox Omni Program.
- Type in "MANAGER" for the password.
- Go to RUN TEST from the menu bar. Scroll down until you see the "Mutatox" test and press run test.
- Change NUMBER OF SAMPLES to "3" and NUMBER OF DILUTIONS to "5".
- The initial concentration is "1.92".
- Press the SET button with an empty cuvette in the READ well. When the Green light comes back on read the cuvette light levels with S-9 test first then the Direct Media.

### 5.3 Test Results Interpretation

Mutatox defines a positive genotoxic response when at lease two dilution tubes show a light increase of 2X or more over the average media control value or two times the appropriate solvent control cuvettes.

## 6.0 QUALITY ASSURANCE/QUALITY CONTROL

Personnel should follow good laboratory practices during Mutatox™ testing.

## 7.0 REFERENCES

Mutatox™ Manual. Microbics Corporation. 1993. Carlsbad, CA. 18 pp.

## 8.0 TABLES

Table 1. Plate Orientation for the S-9 Media Test.
Table 2. Plate Orientation for the Direct Media Test.